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Article

Development and Evaluation of a Multiple Detection System for Diagnosing Malaria and Other Blood Parasitic Diseases in Blood and Stool Samples from Humans and Non-Human Primates

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Abstract: Background/Objectives: Many Tropical diseases such as Malaria, Chagas, Human African Trypanosomiasis, and Lymphatic filariasis coexist in endemic countries, affecting more than 1 billion people worldwide, and are recognized as major global vector-borne diseases. Tackle this disease lies in a correct diagnosis, sensitive, specific, and fast. This study aims to describe and validate a new highly sensitive and specific multiple-analysis system that can effectively detect numerous etiological agents in a single test. **Methods:** A total of 230 human blood samples were evaluated retrospectively for parasite characterization and a total of 58 stool samples from Non-Human Primates (NHP). **Results:** The analytical specificity of the presented method was 100%, with no unspecific amplifications or cross-reactions with other blood parasitic diseases. The detection limit obtained was between 0.6 to 3.01 parasites/μl for *Plasmodium* species, 1.8 parasite/ml for Trypanosomatidae, and 2 microfilariae/ml in the case of Filariae. The sensitivity, specificity, predictive values, and kappa coefficient reached almost 100%, except for the sensitivity for Filariae, which dropped to 93.9%, and its NPV to 89.5%. The operational features described a turnaround and a hands-on time notably shorter than the compared methods with a lower cost per assay. **Conclusions:** This work presents a cost-effective and highly sensitive multiplexed tool (RT-PCR-bp) capable of performing a simultaneous detection for blood parasitic diseases using specific fluorescence probes, enabling the diagnosis of low parasite loads and co-infections.

Keywords: molecular diagnosis; RT-PCR; malaria; Filariae; Trypanosomatidae; neglected tropical diseases

1. Introduction

Many tropical diseases are caused by parasites and transmitted by vectors. Among them is malaria, with 608,000 deaths in 2022 [1], and many of the so-called neglected tropical diseases (NTD; i.e.: lymphatic filariasis, onchocerciasis, Chagas disease, and African human trypanosomiasis) that affect near 1.65 billion people globally [2,3]. The first step for appropriate control lies in a correct diagnosis which, following the World Health Organization (WHO) recommendations, should be sensitive, highly specific, and applicable in endemic areas with limited resources [2,4].

Tropical and subtropical areas of the planet harbour the bulk of parasitic infections. In these regions, coinfections are common due to high prevalence and infection/re-infection rates [3,5].

Control programs for such diseases—including those targeting malaria, African trypanosomiasis, or other specific pathologies—are conducted independently [6,7] without tackling them jointly to optimize available human and technical resources. In clinical cases, and regardless of the setting (endemic or non-endemic), if the patient has fever, malaria tests are performed, and other concomitant pathologies are only looked for if the result is negative, with some exceptions according to the experience of the clinician [8]. Molecular techniques have greatly contributed to the improvement in the diagnosis of multiple diseases, including parasitic diseases. However, most available tests are directed towards individual pathogens, impairing the detection of other sympatric pathogens that can co-infect the same patient. To bridge this limitation, Multiple Analysis Systems (MAS) allow the detection of multiple pathogens in a single test and within a single sample, reducing screening costs and diagnostic turnaround times [9,10]. In addition, a wide range of MAS has been developed to test a variety of biological samples, including faecal, skin, saliva, urine, or blood, as well as for the analysis of genetic material in transmission vectors [11]. However, only a few MAS currently target NTDs.

Here we describe a MAS based on a real-time PCR (RT-PCR) specifically designed for the detection of specific groups of parasites (*Plasmodium* spp., Trypanosomatidae, and Filariae) that, at some stage of their life cycles, are present in blood. The method is carried out in a single process using different primers and specific probes and incorporates an internal reaction control to distinguish between true negatives and potential amplification failures or suboptimal DNA extraction (false negatives).

2. Materials and Methods

The validation of the developed method was carried out by comparing the obtained results against accredited laboratory methods for the detection of these parasites including (i) a nested multiplex PCR for *Plasmodium* spp. detection [12], (ii) a nested PCR for Filariae detection [13], and (iii) an RT-PCR for Trypanosomatidae detection [14]. The evaluation of the MAS was carried out following the recommendations of the Spanish Society of Infectious Diseases and Clinical Microbiology [15]. Performance parameters, including analytical sensitivity and specificity, positive and negative predictive values, analytical sensitivity, reproducibility, and repeatability, were estimated. In addition, we calculate the operational characteristics (turnaround time and economic cost).

2.1. Samples

Two sets of samples were retrospectively investigated in the present study:

- a) Anonymized blood samples ($n = 230$) from returning travelers or immigrants coming from endemic malaria areas were sent to the Parasitology Reference and Research Laboratory at the National Microbiology Centre-Instituto de Salud Carlos III, for the testing of malaria and other tropical diseases. These samples were part of the repository of the Spanish National Biobanks (Registry number: C.0001392) which include the samples used in this study from three research projects for the study of imported malaria in Spain approved by the Ethics Committee of the Instituto de Salud Carlos III and the Research Ethics Committee of the 12 de Octubre University Hospital in Madrid (ISCIII CEI PI 74_2020 Date: 30/09/2020; ISCIII CEI PI 100_2022 Date: 26/01/2022 and H12O CETm: 18/021 Date: 08/02/2022). They consisted of 119 *Plasmodium*-positive samples, including *P. falciparum* ($n = 81$), *P. vivax* ($n = 13$), *P. ovale* ($n = 8$), *P. malariae* ($n = 8$) and mixed infections by two *Plasmodium* species ($n = 9$). In addition, 33 samples for Filariae (*Loa loa*, $n = 24$; *Mansonella perstans*, $n = 7$; mixed infections by *L. loa* + *M. perstans*, $n = 2$), nine samples for Trypanosomatidae (*Trypanosoma brucei*, $n = 4$; *Leishmania infantum*, $n = 3$; *Trypanosoma cruzi*, $n = 2$), and 69 negative samples were also available for the survey.
- b) Faecal samples ($n = 58$) from wild chimpanzees (*Pan troglodytes*) collected in the Dindéfelo National Park (Kedougou, Senegal). These samples were a subset of the initial panel ($n = 234$) originally used to screen for the presence of intestinal and hematic parasites [14]. The animal study protocol was approved by the Research Ethics committee of the Instituto de Salud Carlos III (protocol code CEI PI 90_2018-v2) and the study was conducted in strict accordance with the

Code of Best Practices for Field Primatology of the International Primatological Society [16,17]. They included two *Plasmodium*-positive samples (*P. malariae*, *n* = 1; *Plasmodium* spp., *n* = 1), 16 Trypanosomatidae-positive samples (*T. brucei* sp., *n* =1; *Phytomonas* sp., *n* =8; Trypanosomatidae spp., *n* = 5; *Bodo* sp., *n* = 1; *Neobodo* sp., *n* =1), nine Filariae-positive samples (*M. perstans*, *n* = 8; *Mansonella* spp., *n* = 1), and 31 negative samples.

2.2. DNA Extraction and Purification

Genomic DNA was extracted from 200 µL of whole blood samples collected in EDTA and stored at -20 °C using the QIAamp DNA Mini Blood Kit (QIAGEN®, Hilden, Germany), according to the manufacturer's instructions.

Genomic DNA samples stored at 4 °C were originally isolated from 200 mg of faecal samples preserved in 70% ethanol using the QIAamp DNA Stool Mini Kit (QIAGEN®) according to the manufacturer's instructions.

2.3. Primers and Probes Design

Three PCR primers, one forward and two reverse, were designed in the small subunit ribosomal RNA (ssrRNA) gene sequence for the characterization of the Trypanosomatidae and Filariae families. The forward primer hybridized with both families whereas reverse primers were specific for each one. For the identification of *Plasmodium* spp., two new primers were designed based on the cytochrome oxidase subunit 1 (COI) gene sequence. For the internal reaction control, previously designed primers were used [12]. All primers (Table 1) were designed *in silico* under different cycling conditions such as maximum specificity, alignment temperatures in the range of 60–62 °C, and a maximum GC base content of 40% without self-complementarity, among others [18]. The diagnostic performance of the designed primers was assessed in the laboratory with real human DNA samples.

Table 1. Oligonucleotides used for the molecular detection of the parasites investigated in the present study. Specificity and final concentration values are indicated.

Primer	Sequence (5'–3')	Specificity	Concentration (µM)
JM-U-0011F	CAAGTCTGGTGCCAGCA	Universal	0.2
JM-T-349R	CCAACAAAAGECGAAACGGTGGCC	Trypanosomatidae	0.2
JM-Fi-0015R	CAAGGTAAACTTGCTAGCCAC	Filariae	0.2
JM-P-COI2F	GGTGTGTACAAGGCAACAATAC	<i>Plasmodium</i> spp.	0.2
JM-P-COI1R	CATATAACGGTAAGAAGGTTTCGC	<i>Plasmodium</i> spp.	0.2
IC-Forw	GAGCCGCCTGGATACCGC	Mammals	0.2
IC-Rev	GACGGTATCTGATCGTCTTC	Mammals	0.2
Tryp 681	TxRd–GCTGTTGCTGTAAAGGGTTCGTAG– BHQ2	Trypanosomatidae	0.15
Fi 101	Cy5.5–GGTCCATYCATTGGATGAGAACT– BHQ2	Filariae	0.15
MALCOI 2	Cy5–ATTGGCACCTCCATGTCGTCTCAT– BHQ2	<i>Plasmodium</i> spp.	0.15
IC	Hex–TCGCTCTGGTCCGTCTTG–BHQ1	Mammals	0.06

BHQ1, Black Hole Quencher 1; BHQ2, Black Hole Quencher 2; Cy5, Cyanine-5; Cy5.5, Cyanine-5.5; Hex, Hexachlorofluorescein; IC, Internal Reaction Control; TxRd, Texas Red.

2.4. Real Time PCR for Blood Parasite (RT-PCR-bp) Groups

The RT-PCR-bp reaction mix consisted of 1x Quantimix HotSplit (Biotools, Madrid, Spain) which contained the buffer, the polymerase and dNTPs, the corresponding amount of primers and probes (Table 1), and 5 µl of template DNA in a final reaction volume of 20 µl.

The amplification conditions consisted of an initial denaturation step of five min at 95 °C, followed by 45 cycles of 10 s at 95 °C and 30 s at 60 °C where fluorescence was read in the yellow (excitation peak: 533 nm, emission peak: 559 nm), orange (excitation peak: 596 nm; emission peak: 615 nm), red (excitation peak at 651 nm and an emission peak at 670 nm.), and crimson (excitation peak: 683 nm; emission peak: 703 nm) channels. Amplification was performed in a Rotor-Gene Q 6 plex (QIAGEN®).

All samples were analysed in duplicate and positive controls for each group of parasites, a known negative sample, as well as DNA and No-DNA isolation controls were added to each reaction to detect possible reagent contamination.

2.5. Validation

Validation of the method was performed by direct comparison of obtained results with those from accredited PCR protocols in the laboratory. The reference method for *Plasmodium* detection was a nested multiplex Malaria PCR (SnM-PCR) that identified the four *Plasmodium* species that normally infect humans (*P. falciparum*, *P. malariae*, *P. ovale*, and *P. vivax*) [12]. The reference method for Filariae was a nested PCR (nFil-PCR) that identified, by the size of the amplified fragments, the majority of the Filariae including *Wuchereria bancrofti*, *Loa loa*, *Mansonella perstans*, *M. ozzardi*, *Onchocerca volvulus* and *Dirofilaria* spp. [13]. The reference method for Trypanosomatidae was a RT-PCR (RT-PCR-Tryp), capable of identifying *T. brucei*, *T. cruzi*, *Leishmania* spp., and other kinetoplasts [15]. In case of discrepant results, both tests were repeated. The cases in which the IC was negative were not considered.

2.6. Analytical Sensitivity and Specificity

The analytical sensitivity (or limit of detection, LoD) was calculated in positive samples for each human *Plasmodium* species, for one *T. brucei* sample, and one *L. loa* sample using 10-fold serial dilutions. All samples were tested in duplicates. The initial parasitemia of samples was calculated by microscopy using Giemsa-stained blood smears and following WHO recommendations [19–21] (Table 2).

The LoD was defined as the lowest parasite concentration in which samples and their duplicates were positive [22]. The analytical specificity was determined from the blood samples that were positive for one of the blood parasite groups but negative for the others, detecting possible cross-reactions.

2.7. Intra-Assay Precision

To calculate the intra-assay precision (or repeatability) of the technique, three positive DNA samples for each group of blood parasites with three different levels of parasitemia (high, medium, and low) were analysed by the RT-PCR-bp in three consecutive days and on the same RT-PCR machine [15].

2.8. Inter-Assay Precision

To calculate the inter-assay precision (or reproducibility) of the technique, amplifications of three positive DNA samples for each group of blood parasites with three different levels of parasitemia (high, medium, and low) were carried out on the same day in three different RT-PCR machines [15].

2.9. Performance Parameters

Performance parameters including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy and kappa index were calculated with 95% confidence intervals (95% CI) using Epi Data software version 3.1.

2.10. Operational Features

The processing time was measured from the moment the sample was processed for nucleic acid extraction until the diagnostic result was obtained. Costs per sample were calculated, excluding the expenses for controls included in each run and any duplication of samples. Staff-related costs were estimated based on the time required to perform the techniques. These cost estimates were specific to procedures conducted in Spain; in other countries, kit prices may vary significantly, even between institutions. However, the relative cost differences are expected to remain comparable.

3. Results

3.1. Validation

Primer set concentrations used in the RT-PCR-bp were calculated *in silico* according to temperature. The optimal binding temperature for each set of primers was determined using gradient PCRs in a C1000 Thermal Cycler (BioRad, Berkeley, California, USA). This temperature was set at 60 °C. The optimal concentrations of the probes were determined empirically, being 0.15 µM for all, except for the IC probe that was set at 0.06 µM because mammalian (human or primate) DNA was highly represented in the samples.

3.2. Analytical Sensitivity and Specificity

The LoDs obtained were estimated in the range of 0.6 to 3.01 parasites/µl for *Plasmodium* species, 0.0018 parasites/µl for *T. brucei*, and for 2 microfilariae/ml in the case of *L. loa* (Table 2).

Table 2. Initial parasitemia/microfilaremia levels and calculated limits of detection (LoD) in the serially diluted samples.

Parasite	Initial parasitemia or microfilaremia	LoD
<i>Plasmodium falciparum</i>	73,000 parasites/µl	0.73 parasites/µl
<i>Plasmodium vivax</i>	301 parasites/µl	3.01 parasites/µl
<i>Plasmodium ovale</i>	6,110 parasites/µl	0.61 parasites/µl
<i>Plasmodium malariae</i>	107 parasites/µl	1.07 parasites/µl
<i>Trypanosoma brucei</i>	1,800 parasites/µl	0.0018 parasites/µl
<i>Loa loa</i>	200 microfilariae/ml	2 microfilariae/ml

The analytical specificity was determined in the 102 positive blood samples. Only three blood DNA samples showed co-infections involving *P. falciparum* + *L. loa*. The specific PCRs (SnM-PCR and nFil-PCR) confirmed the results in all three cases, so it was determined that there was no cross-reactivity and that the specificity was 100%.

3.3. Intra-Assay Precision

Repeatability indicates the rate of variation of the method on the same sample when it is used repeatedly under the same conditions and in the same place; in this case for three successive days on the same PCR machine (Table 3).

Table 3. DNA concentration values obtained in the intra-assay precision analysis conducted in the present study. The values indicated the amount of parasites/μl for *Plasmodium* spp. (*P. falciparum* samples) and Trypanosomatidae (*T. brucei* samples) or the amount of microfilariae/ml for Filariae (*Loa loa* samples). Standard deviation and precision values are indicated.

Species	Parasitemia	Day 1	Day 2	Day 3	Standard deviation	Precision (%)
<i>Plasmodium</i> spp.	High	6.98E+04	7.08E+04	7.30E+04	1.64E+03	97.70
	Medium	7.02E+03	7.19E+03	7.30E+03	1.41E+02	98.03
	Low	4.84E+01	4.22E+01	5.36E+01	5.71E+00	88.13
Trypanosomatidae	High	1.84E+04	1.80E+04	1.84E+04	2.29E+02	98.75
	Medium	1.82E+03	1.74E+03	1.68E+03	7.26E+01	95.85
	Low	6.30E+01	8.10E+01	6.30E+01	1.04E+01	84.94
Filariae	High	1.19E+05	1.27E+05	1.19E+05	4.85E+03	96.00
	Medium	2.00E+02	2.03E+02	2.15E+02	7.94E+00	96.15
	Low	9.20E+01	6.60E+01	5.40E+01	1.94E+01	72.51

3.4. Inter-Assay Precision

Reproducibility refers to the percentage of variation in results when different people use the same method in different places or qPCR machines, in this case, it was performed on three different qPCR machines (Table 4).

Table 4. DNA concentration values obtained in the inter-assay precision analysis conducted in the present study. The values indicated the amount of parasites/μl for *Plasmodium* spp. (*P. falciparum* samples), Trypanosomatidae (*T. brucei* samples) and microfilariae/ml for Filariae (*Loa loa* samples). Standard deviation and precision values are indicated.

Species	Parasitemia	M1	M2	M3	Standard deviation	Precision (%)
<i>Plasmodium</i> spp.	High	2.59E+03	2.31E+03	2.01E+03	2.90E+02	87.41
	Medium	3.83E+02	4.30E+02	5.24E+02	7.18E+01	83.89
	Low	2.90E+00	5.01E+00	4.62E+00	1.12E+00	73.12
Trypanosomatidae	High	1.38E+04	1.37E+04	1.24E+04	7.81E+02	94.13
	Medium	2.52E+03	2.61E+03	1.38E+03	6.86E+02	68.40
	Low	1.41E+02	1.44E+02	2.54E+02	6.43E+01	64.16
Filariae	High	3.24E+03	2.83E+03	2.92E+03	2.15E+02	92.81
	Medium	3.12E+02	2.48E+02	3.53E+02	5.29E+01	82.61
	Low	1.72E+01	6.57E+01	2.24E+01	9.54E+00	61.99

M: Real-time PCR machine.

3.5. Diagnostic Performance of the RT-PCR-Bp assay

A concordance of 99.1% (228/230) was achieved between the diagnostic results obtained with the RT-PCR-bp assay and the reference PCR methods of the Parasitology Reference and Research Laboratory (Table 5). Only two Filariae samples were not identified by the RT-PCR-bp assay.

Table 5. Comparison of the diagnostic performance of the RT-PCR-bp and the reference PCR methods for the detection of blood parasites in blood DNA samples (n =230).

	Reference PCR methods	RT-PCR-bp
<i>Plasmodium</i> spp.	119	119
<i>Plasmodium falciparum</i>	81	
<i>Plasmodium vivax</i>	13	
<i>Plasmodium malariae</i>	8	
<i>Plasmodium ovale</i>	8	
Mixed Infections	9	

Trypanosomatidae	9	9
<i>Leishmania infantum</i>	3	
<i>Trypanosoma brucei</i>	4	
<i>Trypanosoma cruzi</i>	2	
Filariae	33	31
<i>Mansonella perstans</i>	7	
<i>Loa loa</i>	24	
Mixed Infections	2	
Negative	69	71
Total	230	230

The RT-PCR-bp assay showed sensitivity, specificity, PPV, and NPV values of 100% for *Plasmodium* spp. while for Filariae these values ranged from 89.5% to 100% (Table 6). In the case of Trypanosomatidae, these values couldn't be statistically calculated due to the low sample number, although 100% of the samples gave the expected result.

Table 6. Performance parameters of the RT-PCR-bp developed in the present study. 95% Confidence Intervals (95% CI) and Kappa index values are indicated.

	<i>Plasmodium</i> spp.			Filariae	
	Values (%)	95% CI		Values (%)	95% CI
Sensibility	100	94.0–100		93.9	80.4–98.3
Specificity	100	81.6–100		100	81.6–100
PPV	100	94.0–100		100	89.0–100
NPV	100	81.6–100		89.5	68.6–97.1
Kappa index	1.00	1.00–1.00		0.91	0.80–1.03

NPV: negative predictive value; PPV: positive predictive value.

Of the 58 stool DNA samples, 34 were positive by RT-PCR-bp (58.6%) while previously only 27 (46.5%) samples tested positive in the study of Köster et al., 2021. In addition, the RT-PCR-bp assay allowed the identification of 9 mixed infections that were missed by the reference PCR methods (Table 7). In Trypanosomatidae, three expected positive cases were negative, whereas four new positive cases were detected.

Table 7. Comparison of the diagnostic performance of the RT-PCR-bp and the reference PCR methods for the detection of blood parasites in faecal DNA samples ($n = 58$).

	<i>Plasmodium</i> spp.	Trypanosomatidae	Filariae	Coinfected	Total infected
RT-PCR-bp n (%)	12 (21)	17 (29)	14 (24)	9 (16)	34 (58)
Reference PCR method n (%)	2 (3.4)	16 (27.6)	9 (15.5)	3 (5.2)	27 (46.5)

Reference PCR method: study of Köster et al., 2021.

3.6. Operational Features

The estimated turnaround time to complete the process—from sample processing to the provision of results—was 3 h and 30 min for the RT-PCR-bp. In comparison, the turnaround time for the reference methods was approximately the same for the RT-PCR for Trypanosomatidae, but significantly longer for the conventional SnM-PCR and nested Filariae PCR (Table 8).

The hands-on time was notably shorter for the RT-PCR-bp, involving only sample preparation, PCR setup, and result analysis, amounting to approximately 1 h. For the reference methods, the hands-on time increased significantly due to the need to prepare five separate PCRs, including the two conventional nested PCRs, and conduct the corresponding electrophoretic procedures, totalling approximately 5 h.

Regarding costs per sample, for the RT-PCR-bp, the amplification kit, probes, and primers must be considered, assuming approximately €6 per reaction. For the RT-PCR of Trypanosomatidae, the cost was slightly lower at €5 per reaction, as it uses only two probes instead of four. For the two conventional nested PCRs, the cost is around €2 per sample, including the amplification kits, dNTPs, primers, and electrophoresis components. Overall, the total cost for the reference methods amounts to €9 per sample.

Table 8. Estimated turnaround time to complete the process—from sample processing to the provision of results.

	RT-PCR-bp	SnM-PCR	nFil-PCR	RT-PCR-Tryp
Sample Processing	1h	1h	1h	1h
First PCR setup	30 min	30 min	30 min	30 min
First PCR amplification	1h 30min	2h	2h	1h 30min
Second PCR setup	-	30 min	30 min	-
Second PCR amplification	-	1h 15min	1h 15min	-
Electrophoresis	-	30 min	30 min	-
Results analysis	30 min	15 min	15 min	30 min
Total	3h 30min	6h	6h	3h 30min

Sample processing includes DNA extraction and purification. PCR setup includes master mix and PCR preparation.

4. Discussion

Parasitic diseases, including malaria, human African trypanosomiasis, Chagas disease, and lymphatic filariasis—among other NTDs—continue to threaten over 1 billion people worldwide and are recognized as major global vector-borne diseases [3,23]. Despite the implementation of several control strategies and programs in tropical countries, the complex life cycles of parasites, the existence of animal reservoirs, and the co-endemicity of multiple pathogens underscore the urgent need for new control approaches. These approaches should incorporate MAS-based methods, instead of single detection systems such as rapid diagnostic tests or loop-mediated isothermal amplification (LAMP) [24], which offer highly sensitive and multiplexed screening for several pathogens in a single test [10].

This work presents a cost-effective and highly sensitive multiplexed tool (RT-PCR-bp) that can effectively and simultaneously detect *Plasmodium* spp., Trypanosomatidae, and filarial worms using specific fluorescence probes, allowing the detection of low parasite loads and co-infections. An asset of the RT-PCR-bp is the incorporation of an internal reaction control for differentiating DNA extraction errors and amplification failures from true negative results. Furthermore, no unspecific amplifications or cross-reactions among the three groups of blood pathogens tested here were observed, proving the diagnostic usefulness of this method in detecting mixed infections.

The RT-PCR-bp merges in a single reaction, a previously designed RT-PCR for Trypanosomatidae, and a transformation of a conventional PCR into an RT-PCR for the detection of filarial worms with the design of a new specific probe [14]. Both assays were based on the amplification of the *ssrRNA* marker. In addition, two new primers and their corresponding specific probe have been designed based on the mitochondrial COI gene for the specific detection of *Plasmodium* spp.

According to the results obtained, whereas the analytical specificity was 100%, the only observed variation for the three groups of blood parasites was related to the reproducibility precision values due to each equipment's inherent limitations in quantifying low parasite loads. It is important to note that these parameters are directly related to each equipment and to their correct maintenance and calibration, and not to the assay itself. However, both parameters provided this method as a reliable tool, with every sample correctly detected as positive for each group of parasites and each parasite load in every essay.

Besides, the statistical values showed that this method is comparable with our previously mentioned reference methods and the analytical sensitivity, with detection limits of 0.73–3.01 parasites/ μ l

for *Plasmodium* spp. (depending on the species), 0.0018 parasites/ μ l for *T. brucei* and 2 microfilariae/ml, aligns with other established methods. For malaria, the LoD is comparable to those reported by other researchers using Nested PCR, RT-PCR, and LAMP [12,13,24,25]. However, the RT-PCR-bp method demonstrates a LoD one logarithm lower for the detection of *P. ovale* (0.61 parasites/ μ l), compared to 0.21 and 2.3 parasites/ μ l in LAMP and RT-PCR, respectively [24,25]. These discrepancies may be attributed to differences in the *P. ovale* subspecies analysed, as previous studies did not differentiate between them [24]. Overall, this method showed a general sensitivity for *Plasmodium* spp., which corresponds favourably to other published RT-PCR methods with detection limits around 1p/ μ l [26]. In Trypanosomatidae, various studies have reported LoDs ranging from 0.1 parasites/ml in the case of *T. brucei* [27,28], to 0.5 and 5 p/ml depending on the species [29,30] and genotype of *T. cruzi* [29–31], similar to the obtained with the current method (1.8 parasite/ml). For filariasis, the detection limit depends on the species, as shown by other authors [32], for *Mansonella perstans* microfilaremia below 0.61 μ f/ml is not detected, while parasitemias over 2.5 μ f/ml are detected independently of the species [32]. The LoD of the RT-PCR-bp was 2 μ f/ml, which correlates with the previous study.

A key aspect of this method is the detection of mixed infections by [29–31] two or more parasite groups. Initially, samples corresponding to mixed infections between groups had not been included since these samples had arrived at the Reference Laboratory to diagnose or confirm a single pathology, but when applying the method, three mixed infections of *Plasmodium* spp. and filarial worms were characterized. Likewise, 16% of coinfections were observed in the faecal samples of the chimpanzees. This shows the importance of multiple detection systems to perform a correct diagnosis of mixed infections and here, molecular methods are essential due to their greater sensitivity and specificity compared to microscopy [25]. Misdiagnosis of mixed infections may involve inadequate treatment, damaging the patient's health and the control of the disease endemic areas [25].

It is important to note that faecal samples are not optimal biological samples for detecting blood parasites such as *Plasmodium* spp., Filariae, or *Trypanosoma* spp., which are more accurately identified in blood samples. However, other studies have shown the presence of these parasites in non-human primate faeces [33–36]. In this study, the value of using these samples was the comparison with the previous published results by Köster et al. (2021) [14]. The new RT-PCR-bp incorporates the Trypanosomatidae detection PCR (RT-PCR-Tryp) used in that work, so no improvements were expected. On the contrary, in the case of Filariae, where a conventional nested PCR method has been changed to an RT-PCR, the percentage of samples infected with Filariae has gone from 15.5% to 24.0%. In malaria, the increase is still greater, from 3.4% to 21.0%. This could be attributed not only to the switch from conventional PCR to RT-PCR but also to the change in target from the ssrRNA to the mitochondrial COI gene.

Regarding operational characteristics, the time required to obtain results was significantly shorter with RT-PCR-bp compared to the reference methods. This efficiency is also evident in the reduced staff working time, with the reference methods requiring approximately 4 additional hours. Moreover, the cost of RT-PCR-bp is lower than the combined price of all the reference methods. Therefore, it is recommended to use the RT-PCR-bp assay as a screening tool. For positive cases, the corresponding PCR assays can then be employed to identify the species involved in the infection or to sequence the amplified fragments.

The limitations of this study are related to the number of samples available for the different pathologies, especially in the cases of Trypanosomatidae and mixed infections. Another limitation is related to costs since the prices of amplification kits, probes and primers vary between countries and those reflected here may not be extrapolated.

5. Conclusions

Overall, the RT-PCR-bp method presented here demonstrated high sensitivity, specificity, and cost-effectiveness as a multiple analysis system capable of the simultaneous detection of *Plasmodium* spp., Trypanosomatidae, and filarial parasites. This approach enables the diagnosis of low parasite

loads and co-infections, facilitating appropriate control measures for multiple etiological agents in a single test. Consequently, it contributes to timely and improved diagnosis and treatment.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, laboratory samples came from the repository of the Parasitology Reference and Research Laboratory (Collection Number: C.0001392. Spanish National Biobanks Registry) which include the samples used in this study from three research projects for the study of imported malaria in Spain approved by the Research Ethics Committee of the Instituto de Salud Carlos III and the Research Ethics Committee of the 12 de Octubre University Hospital in Madrid (ISCIII CEI PI 74_2020 Date: 30/09/2020; ISCIII CEI PI 100_2022 Date: 26/01/2022 and H12O CETm:18/021 Date: 08/02/2022). The animal study protocol was approved by the by the Research Ethics committee of the Instituto de Salud Carlos III (protocol code CEI PI 90_2018-v2) and following the Guidelines of Best Practices for Field Primatology of the protocols of the International Primatological Society.

Informed Consent Statement: Human samples are sent to the laboratory either as part of research projects—accompanied by informed consent that allows their storage in a biobank or collection after the project concludes (protocol Ethics Committee of the Instituto de Salud Carlos III CEI PI 74_2020 Date: 30/09/2020; Ethics Committee of the Instituto de Salud Carlos III CEI PI 100_2022 Date: 01/26/2022 and Ethics Committee of the 12 de Octubre University Hospital in Madrid CETm:18/021 Date: 02/08/2022) —or for diagnostic purposes. In the latter case, the samples are anonymized and stored in a collection, to be used following the ethical standards established by the Ethics Committee of the Instituto de Salud Carlos III, aimed at improving and validating diagnostic methods related to the initial diagnostic suspicion. In both scenarios, once the embargo period has ended or the research project is completed, the samples are stored in the laboratory's Spanish National Biobanks Registry sample collection Number: C.0001392.

Data Availability Statement: All data are available upon request (jmrubio@isciii.es).

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